MICROSATELLITE MARKERS ISOLATED FROM THE WILD MEDICINAL PLANT CENTELLA ASIATICA (APIACEAE) FROM AN ENRICHED GENOMIC LIBRARY

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Microsatellite markers isolated from the wild medicinal plant *Centella asiatica* (Apiaceae) from an enriched genomic library[1]

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- *Premise of the study:* Microsatellite markers for *Centella asiatica*, an important medicinal herb, were developed and characterized to promote genetic and molecular studies.
- *Methods and Results:* A GA/GT-enriched genomic library was constructed from an accession from Madagascar. Roughly 75% of the 768 clones of the enriched library contained microsatellites. Eighty sequences containing microsatellites were obtained from 96 positive clones. Specific primers were designed for 20 loci, and 17 of them displayed polymorphism when screened across 17 *C. asiatica* accessions, with an average of 4.3 alleles per locus. The observed and expected heterozygosity values averaged 0.114 and 0.379, respectively.
- *Conclusions:* This is the first report constructing an enriched genomic library and identifying microsatellite markers from *C. asiatica*. These 17 polymorphic microsatellite markers are a useful resource for this plant, applicable for diversity studies, pedigree analyses, and genetic mapping.

Key words: Apiaceae; *Centella asiatica*; enriched genomic library; Madagascar; microsatellites; simple sequence repeat markers.

Centella asiatica (L.) Urban (Apiaceae) is an important medicinal herb that is used in both traditional pharmacopoeia and modern medicine. Asiaticoside is one of the principal compounds commercially used as a wound-healing agent, owing primarily to its anti-inflammatory effects. Madecassol and Blastoestimulina are the most notable pharmaceutical products derived from this plant (Randriamampionona et al., 2007). *Centella asiatica* is a perennial, herbaceous creeper, mainly found in tropical and subtropical countries. Madagascar is the world's top *C. asiatica* producer, and the species is second only to Madagascar periwinkle (*Catharanthus roseus*) as a medicinal plant export (Péchard et al., 2005). Chemical, medicinal, and pharmacological studies have been conducted to better understand the medicinal properties of pentacyclic triterpenoids from *C. asiatica* (Pittella et al., 2009); however, little is known about its biology and genetics. This plant has been reported to be diploid with $2n = 2x = 18$, but some older controversial studies questioned either the number of chromosomes, the presence of B chromosomes, or ploidy level (Das and Mallick, 1991). Sequence data are restricted to some expressed sequence tags, mRNA, and PCR-amplified gene fragments, which have been used for phylogenetic analysis of the Apiaceae (Downie et al., 2000). In this paper, we report the development and characterization of microsatellite markers for *C. asiatica* that can be used for taxonomic, diversity, and phylogenetic studies of this species.

METHODS AND RESULTS

An accession from Vohimana (Appendix 1), in eastern Madagascar, was used to construct a microsatellite-enriched library following the protocol described by Billotte et al. (1999). Ten micrograms of genomic DNA were digested with *Rsa*I endonuclease and ligated to RSA21 and RSA25 adapters. Ten nanograms of ligated DNA were amplified with RSA21 primer and purified. The PCR products were submitted to a hybridization-based capture with 5′ biotine-labeled microsatellite oligoprobes $I5(GA)_8$ and $I5(GT)_8$ using streptavidine-coated magnetic beads (Promega, Madison,Wisconsin, USA). The eluted fraction was PCR amplified with RSA21 primer, cloned into pGEM-T Easy vector (Promega), and then transformed into DH10BT1R *Escherichia coli* strain (Invitrogen, Carlsbad, California, USA). Inserts from white colonies were PCR amplified, separated onto 1.2% agarose gel, transferred onto Hybond-N+ nylon membranes (GE Healthcare, Fairfield, Connecticut, USA), and hybridized at 56°C with [γ32P] ATP 5′ end-labeled $(GA)_{15}$ and $(GT)_{15}$ probes.

Among 768 clones of the microsatellite-enriched genomic library of *C. asiatica*, 560 contained simple sequence repeat (SSR) markers, giving an enrichment rate of 73%. Ninety-six of these clones were selected based on the strength of hybridization signal and sequenced using a 96-capillary 3730xl DNA analyzer (Applied Biosystems, Foster City, California, USA) at GATC Biotech Company (Konstanz, Germany). We analyzed the sequences and designed primer

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pairs with the dedicated pipeline SAT (Dereeper et al., 2007), using default parameters proposed for enriched libraries. We obtained 76 singletons and four clusters with more than two sequences. Thirty-seven sequences (46.25%) contained compound dinucleotide motifs (i.e., combination of AG, GT, and AT motifs), 32 sequences (40%) harbored simple dinucleotide repetitions, and 11 sequences (13.75%) harbored compound complex di-, tri-, tetra-, hexa-, and pentanucleotide motifs. Primer pairs were designed for 30 SSR markers. All gave amplification products on the reference plant DNA and also on DNA extracted from the putative closely related species *C. uniflora*. Only 20 SSR markers were repeatable and easy to score, and were conserved for further polymorphism study.

To assess microsatellite polymorphism, DNA from 17 accessions was used: 16 *C. asiatica* accessions collected in Madagascar and one accession from India (see Appendix 1 for precise locations). Characteristics of the 20 SSR markers are shown in Table 1. PCR amplifications were performed in a final volume of 10 μL containing 25 ng of DNA as template, following the PCR protocol of Oblessuc et al. (2009) and resolved on an ABI3500xl DNA sequencer (Applied Biosystems). Seventeen markers were polymorphic and subsequently analyzed; the remaining three were monomorphic. A total of 73 alleles were produced at the 17 polymorphic loci, with an average of 4.3 alleles per locus. Four SSR markers (mCaCIR004, mCaCIR009, mCaCIR019, mCaCIR024) showed low polymorphism with two alleles per locus, and the rest were polymorphic with more than four alleles per locus (Table 2). No difference of polymorphism level could be observed between SSRs derived from pure dinucleotide, compound, or complex motifs. Genetic analysis was carried out using PowerMarker software 3.25 (Liu and Muse, 2005). Observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated according to the formula of Nei (1973); mean H_o was 0.114 (range: 0–0.625) and mean H_e was 0.379 (range: 0.218–0.585) (Table 2). Significant linkage disequilibrium was found for 114 marker pairs of the 136 possible pairs.

CONCLUSIONS

The values obtained for H_o and H_e correspond to SSR loci with a medium level of polymorphism. Discrepancy between H_o and H_e could be explained by null alleles, inbreeding, or by the small sample size. Similarly, the significant linkage disequilibrium reported here might reflect either significant genetic differentiation among populations of *C. asiatica* or strong genetic linkage between markers. Nevertheless, taking into account the small size of the sample, more data are required to confirm the cause of linkage disequilibrium, and new insight into the biology of *C. asiatica* is needed to clarify

Table 1. Characterization of 17 microsatellites in *Centella asiatica*.

SSR locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	EMBL accession no.
mCaCIR002	F: CCACAGGTAACACCGAAT R: GCACTTGCACTATCTGGAA	$(CT)_{30}$	141–169	55	FR838931
mCaCIR004	F: GGGTGGTCTGCCTAAAGA R: TGGAGATCAAGTTTCATGC	$(CT)_{17}$	190–202	56	FR838932
mCaCIR005*	F: GGCCTTCAATGTATGCTG R: TTTGATTTGTTGGGTCTTG	$(GA)_{16}$	170	55	HE605293
mCaCIR006	F: ACGGGCATTTATTCCATT R: GCAAACCACCACAACTTC	$(CT)_{15}$	215–219	55	FR838933
mCaCIR007	F: TGGAGGTGGTGTAACTGG R: AGGGGATCAAACCTCATC	$(TG)_{12}$	234–240	55	FR838934
mCaCIR009	F: TGCCTATCCTTTGAATGC R: CAAACATGACATTCTTAAAACA	$(TC)_{11}$	289–295	55	FR838935
mCaCIR010	F: AATGTAAAATTCCCGGTGT R: TAAACAGGCGTTCCAAGT	$(TG)_{10}$	233–251	54	FR838936
mCaCIR011	F: TTCATAAAAGTCCTTCCACA R: TAGGTTGATGTGGCCTCT	$(CA)_{10}$	215–217	53	FR838937
mCaCIR012	F: CACGAAAATTGGAAACAA R: CATGTGAGTTTATGAGTTTCTATG	$(AC)_{10}$	207–217	53	FR838938
mCaCIR013*	F: CAAGTTCCTCCCACGAAT R: GCCGAAATAATCGAAATATAAG	$(AC)_9$	201	55	HE605294
mCaCIR018	F: TTGAGTTTAAGAAGTCCCAAAT R: AATCCTTCACACTCCTAAAGC	$(TC)_{23}(TA)_7$	174–221	55	FR838939
mCaCIR019	F: TTTCTTGTTAAATGCGATGA R: AATGACATCACTGCTATGGA	$(GA)_{15}(GTGC)_3$	206–212	54	FR838940
mCaCIR020	F: TTTAGGAAGTTGGATTTTGC R: GGTTTAATTCAGGACGCTTA	$(AC)_7(AC)_5$	174–194	55	FR838941
mCaCIR021*	F: TGCCTAGATTTTGGGTTTT R: TCTTACAATGCAATCAACCT	$(CT)_{24}(ATT)_4$	200	55	HE605295
mCaCIR022	F: AGGAGTATTGACAAGAGGTGA R: GGATGGCAGTCCATTTTA	$(CT)_6(CT)_{11}(CA)_9$	245–261	54	FR838942
mCaCIR024	F: TCTTTCGTTGATACATGCAC R: AAAACTTAAAGAAGATACAAACTCC	$(ATTA)_3$	241–275	55	FR838943
mCaCIR027	F: ACCCCAAGACCTTCAGTT R: CCTTCTGCTTTCCCTTTT	$(CA)_6(AG)_{10}$	225–233	54	FR838944
mCaCIR028	F: CAGAGTTTGGGCAGAAAA R: GACGAGTGGAGGATAAGAAA	$(AG)_5(AG)_8$	197–203	55	FR838945
mCaCIR029	F: GGTCTGAGGTCTGTTGAGG R: CGCATTGACAGAACAAAA	$(CA)_8(AT)_5(CT)_5$	317–366	55	FR838946
mCaCIR030	F: GGCAAATCGAGAGCAATA R: ACGGAAAAGCCTAACAGC	$(TG)_5(TA)_5(GA)_{20}$	240–248	55	FR838947

Note: EMBL = European Molecular Biology Laboratory; T_a = annealing temperature.
* Indicates monomorphic marker.

TABLE 2. Genetic data for 17 accessions of *Centella asiatica* using the polymorphic microsatellite markers developed in this study.

SSR locus	N_a	H_o	H_e
mCaCIR002	5	0	0.226
mCaCIR004	3	0	0.32
mCaCIR006	4	0	0.218
mCaCIR007	6	0.063	0.377
mCaCIR009	3	0	0.218
mCaCIR010	6	0.625	0.585
mCaCIR011	4	0.188	0.365
mCaCIR012	4	0.063	0.279
mCaCIR018	5	0.125	0.527
mCaCIR019	2	0	0.226
mCaCIR020	4	0.063	0.369
mCaCIR022	5	0	0.5
mCaCIR024	3	0.25	0.576
mCaCIR027	5	0.188	0.541
mCaCIR028	4	0	0.219
mCaCIR029	5	0.25	0.494
mCaCIR030	5	0.125	0.414

Note: H_e = expected heterozygosity; H_o = observed heterozygosity; N_a = number of alleles.

ploidy and reproductive biology, before these SSR markers can be used for population genetic studies. Despite these potential limitations, a wide range of plant genetic studies can now be envisaged, such as diversity studies and pedigree analyses. Genetic mapping, which requires a higher number of markers, is now possible based on this microsatellite-enriched library of *C. asiatica*, which potentially contains hundreds of SSRs. Finally, the cross-species amplification observed here will allow phylogenetic analyses and clarify taxonomic status within *Centella*.

APPENDIX 1. Voucher information for the 17 accessions from Madagascar used to assess microsatellite polymorphism of *Centella asiatica*. Voucher specimens are deposited at Tsimbazaza Herbarium (TAN), Madagascar; five samples were collected per location.

Region	Collection location	Altitude (m)	Longitude (°E)	Latitude (°S)
Antananarivo	Ambohitromby	803	46.273888	17.989167
Antananarivo	Tsiroanomandidy	940	46.059816	18.754022
Antananarivo	Belobaka	820	45.697253	18.993603
Antananarivo	Mandoto	1475	46.311173	19.573930
Diego	Ambanja	5	48.428727	13.721140
Mahajanga	Antsohihy	21	48.049614	14.912999
Mahajanga	Besalampy I	39	44.493775	16.746286
Mahajanga	Kandreho	199	46.091667	17.485277
Mahajanga	Ambatomainty	438	45.991111	17.416944
Mahajanga	Maintirano	26	44.033056	18.063687
Mahajanga	Besara Sahoany	55	44.287228	18.684945
Mahajanga	Antsalova	117	44.619426	18.681393
Mahajanga	Tsiandro	730	44.900000	18.730000
Toamasina	Vohimana	963	48.836877	18.957978
Toliara	Miandrivazo	154	45.454100	19.532300
Toliara	Beroboka	47	44.607971	19.966997

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